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16. ABSTRACT

A monoclonal antibody, 3F7, which reacts with the common rotavirus antigen on the sixth viral gene product was prepared. It was used in a direct monoclonal antibody radioimmunoassay (RIA) as a diagnostic reagent for detection in 3.5 hours of rotavirus in human pediatric stool speeimens. In the 177 samples tested, a concordance of 96 percent was seen between the monoclonal RIA and the well-established and commonly used commercially available Rotazyme test. Six discrepant specimens which were positive by monoclonal RIA but negative by Rotazyme were shown to be positive by either electron microscopy or confirmatory blocking immunoassay. A seventh discrepant specimen was positive by Rotazyme and negative by monoclonal RIA as well as by both direct and immune electron microscopy. The monoclonal RIA test appears to be highly sensitive and specific, and merits additional evaluation as a rapid, convenient diagnostic assay that can reduce currently encountered problems associated with diagnosing rotavirus by immunoassay.

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Detection of Rotavirus in Human Stools by Using Monoclonal Antibody

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A monoclonal antibody, 3F7, that reacts with the common rotavirus antigen on the sixth viral gene product was prepared. It was used in a direct monoclonal antibody radioimmunoassay (RIA) as a diagnostic reagent for detection, in 3.5 h, of rotavirus in human pediatric stool specimens. In the 177 samples tested, a concordance of 96% was seen between the monoclonal RIA and the well-established and commonly used commercially available Rotazyme test. Six discrepant specimens that were positive by monoclonal RIA but negative by Rotazyme were shown to be positive by either electron microscopy or confirmatory blocking immunoassay. A seventh discrepant specimen was positive by Rotazyme and negative by monoclonal RIA as well as by both direct and immune electron microscopy. The monoclonal RIA test appears to be highly sensitive and specific, and merits additional evaluation as a rapid, convenient diagnostic assay that can reduce currently encountered problems associated with diagnosing rotavirus infection by immunoassay.

Rotavirus is a major cause of pediatric gastroenteritis and is responsible for about half of the cases of acute diarrheal illness among hospitalized patients 6 to 24 months of age (5, 17; G. Cukor and N. R. Blacklow, Microbiol. Rev., in press). Rotavirus illness is also common in community and family settings (14). A rapid diagnosis of this infection may be valuable in the formulation of a prognosis for children with acute diarrhea and may prevent the unnecessary use of antibiotic therapy and prolonged hospitalization. Rapid diagnosis also allows for the timely institution of appropriate infection control measures in hospitals, and for the gathering of epidemiological and clinical data that are vital for vaccine development.

Although in vitro cultivation of human rotavirus from stools has now been achieved (3, 19, 24, 27, 29), it is not a practical diagnostic technique because of relatively low isolation rates and the frequent need for "blind" passages in cell cultures. The diagnostic test of choice in most settings is the solid-phase immunoassay, in preference to the less practical electron microscopy (EM). Immunoassay offers the possibility of same-day or next-day diagnosis and can be performed on a large scale with routinely available equipment and personnel (9). The most widely used commercially available test for rotavirus, and the only one with which there is wide published experience (2, 7, 15, 16, 23), is the Rotazyme enzyme immunoassay (Abbott Laboratories, North Chicago, Ill.). It has been found to be equal to or slightly less sensitive than EM for detecting rotavirus. Rotazyme has not been noted to suffer from problems of nonspecificity, except in the testing of neonatal specimens (18). The Rotazyme test is based on the observation that hyperimmune antiserum raised against the readily cultivated and purified simian rotavirus, SA11, is useful as a diagnostic reagent for human rotavirus (8). This is because serotypes of human rotavirus share a common group-specific antigen with rotaviruses of other mammalian hosts.

In this report we present a new, rapid immunoassay for detection of rotavirus in human clinical specimens. This assay utilizes for the first time as a clinical diagnostic reagent a defined monoclonal antibody directed against the common group-specific rotavirus antigen. We have evaluated our test by comparison with the well-established and widely used Rotazyme assay.

MATERIALS AND METHODS

Stool specimens. A total of 177 stool specimens were obtained from pediatric patients, ages 2 months to 11 years (median age, 19 months), who were seen for acute diarrheal illness. One hundred fourteen of these samples were collected in New England during 1982 to 1983. They were obtained at the University of Massachusetts Medical Center, Worcester (97 specimens), the University of Connecticut, Farmington (11 specimens), and the Massachusetts General Hospital, Boston (6 specimens). The remaining 63 specimens were collected in the Far East in 1975 and 1976 (11, 12). All samples were stored at −70°C. Before testing, stool specimens were thawed and a 2 to 5% suspension in veal infusion broth was prepared as described previously (4). Seven stool specimens were submitted under code for direct and immune EM examination for rotavirus by Carl Brandt of the Washington, D.C. Children's Hospital (5).

Viruses. A stock of murine rotavirus (EDIM) was prepared by inoculation of 5-day-old mice as previously described (28). The cultivation of simian rotavirus SA11 in monkey kidney cells and its sucrose gradient purification have also been described (8). Culture-adapted human rotavirus Wa was obtained from R. G. Wyatt (30). The Cody strain of bovine rotavirus NCDV was grown as described previously in primary bovine embryonic kidney cells (10).

Preparation of monoclonal antibody. For monoclonal antibody production, a 2-month-old BALB/cJ mouse was inoculated intraperitoneally with 3×10^5 50% mouse infectious doses of EDIM mixed with Freund complete adjuvant. The inoculation was repeated 3 weeks later, and a month after that, 10^5 50% infectious doses of EDIM were given intravenously without adjuvant. Three days later, the mouse was sacrificed and its spleen was removed for fusion. Serum antibody levels of the mouse to both EDIM and SA11 virus were monitored by radioimmunoassay (RIA) (28), and rose from a preimmunization level of <1:50 to a titer of 1:3,200

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for each virus at the time of sacrifice. The isolated murine spleen cells were fused in a 10:1 ratio with SP2/0 mouse myeloma cells in the presence of 40% polyethylene glycol. Hybridoma cultures were established and maintained as described by Greenberg et al (13). Culture supernatants were screened for the presence of antibody to the rotavirus group-specific antigen by RIA reactivity with purified SA11 virus (28). Antibody-positive cultures were then cloned by limiting dilutions on a feeder layer of murine thymocytes. Monoclonal antibodies of interest were concentrated by affinity chromatography on a staphylococcal protein A-Sepharose resin (20). The isotype of the monoclonal antibody was determined by a commercially available test (Monoab EIA kit, Zymed Laboratories, San Francisco, Calif.). The specificity of the monoclonal antibody was determined by immunoprecipitation of a ^{35}S-labeled, SA11-infected cell lysate (13, 20).

Preparation of polyclonal antisera. A young adult New Zealand white male rabbit was immunized with three intramuscular injections of purified SA11 virus. The inoculations, spaced 3 weeks apart, each contained 10^8 50% tissue culture infectious doses of virus mixed with Freund incomplete adjuvant. Three weeks after the final inoculation, a serum, SR3, was obtained that had an anti-SA11 RIA titer of 1:64,000 (4). The immunoglobulin (IgG) fraction of SR3 was prepared by ammonium sulfate precipitation and ion-exchange chromatography and was radioiodinated by the chloramine T procedure as previously described (4). A previously prepared anti-SA11 guinea pig serum was used as a blocking reagent (8).

"Multideterminate" RIA test, using monoclonal antibody. Wells of a polyvinyl chloride microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl of a 1:10,000 dilution of pre- or postimmunization (SR3) anti-SA11 rabbit serum for 4 h at 37°C. The plates were washed twice with phosphate-buffered saline containing 0.1% azide (PBSZ). For postcoating, the wells were filled overnight with gel saline consisting of PBSZ with 1% swine skin gelatin (Sigma Chemical Co., St. Louis, Mo.). In a separate tube, equal volumes of the test stool suspension and a solution of purified monoclonal antibody, 3F7, in gel saline (4 μg of antibody per ml) were mixed and incubated for 15 min at 37°C. The plates were washed three times with PBSZ, and 50 μl of the stool-antibody mixture was added per well for 2 h at 37°C. The plates were washed five times with PBSZ containing 0.05% Tween 20. This was followed by the addition of 200,000 cpm of freshly ^{125}I-labeled rabbit anti-mouse immunoglobulin (Cappel Laboratories, Malvern, Pa.) per well for 2 h at 37°C. The plates were washed five times with PBSZ-Tween, and the wells were cut apart. Bound radioactivity was determined in a gamma counter. A sample was considered positive if the counts bound to the SR3-coated well divided by the counts bound to the control preimmunization serum-coated well (P/N ratio) was ≥2. Optimal test conditions for this assay, as well as for the direct monoclonal antibody test, were determined by extensive checkerboard titration.

Direct monoclonal antibody test. The direct test differed from the multideterminate assay in that wells of a microtiter plate were coated with 100 μl of purified 3F7 monoclonal antibody (4 μg/ml in PBSZ); other wells were coated with an irrelevant (anti-rat T cell) control monoclonal antibody at the same concentration or with gel saline. Antibody-coated microtiter wells were stable for at least 1 week at 4°C but were not studied for longer periods for their stability characteristics. Postcoating was accomplished as described above. Tween 20 was added to stool suspensions before testing to a final concentration of 0.05%, and 50 μl of each sample was added per well for 1 h at 37°C. Plates were washed five times with PBSZ-Tween, and 200,000 cpm of freshly labeled ^{125}I-labeled SR3 immunoglobulin was added per well for 2 h at 37°C. In this test, the counts bound to the control wells remained near background levels, and a P/N ratio of ≥5 was considered positive. For the confirmatory blocking test, the RIA reactions of stool specimens preincubated for 1 h at 37°C with either pre- or postimmunization anti-SA11 guinea pig serum (8) were compared.

RESULTS

Characterization of monoclonal antibody to rotavirus. Hybridoma clone 3F7 was chosen as a candidate diagnostic reagent. The culture showed characteristics of rapid growth, stability during storage in liquid N_2, and stable antibody production. Monoclonal antibody 3F7 showed high RIA reactivity (P/N values > 5) not only with SA11 virus but also with EDIM, culture-adapted human rotavirus Wa, the bovine rotavirus NCDV, and a screening panel of six rotavirus-positive human stools. 3F7 showed no RIA reactivity with an extract of mouse gut, simian cells (MA104), or a panel of six rotavirus-negative human stools. Monoclonal antibody 3F7 is IgG 2a kappa. It is stable to concentration, purification, and storage at −20°C. The antibody binds satisfactorily to polyvinyl chloride microtiter wells. Immunoprecipitation was used to demonstrate that 3F7 reacts with VP6, the 42,000-dalton product of the sixth rotaviral gene (Fig. 1). VP6 contains the common group-specific rotaviral antigen (13).

Monoclonal antibody tests for rotavirus. Monoclonal antibody 3F7 and anti-SA11 rabbit serum SR3 were utilized in diagnostic tests of two different configurations. In the multideterminate assay test (31), microtiter wells were coated with SR3 and other wells were coated with control preimmunization rabbit serum. Stool specimens were mixed with

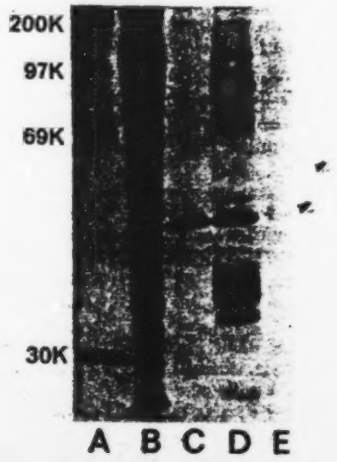

FIG. 1. Immunoprecipitate of ^{35}S-labeled, SA11-infected cell lysate was analyzed by polyacrylamide gel electrophoresis by the method of Rogan et al. (22). ^{14}C-labeled molecular weight markers (New England Nuclear Corp., Boston, Mass.) were myosin, phosphorylase B, bovine albumin, and carbonic acid. Lane A: molecular weight markers; lane B: ^{35}S-infected cell lysate; lanes C, D, and E represent immunoprecipitates with monoclonal antibody 3F7 (C), anti-SA11 guinea pig serum (D), and SP2/0 control supernatant (E).

3F7 and incubated. The stool-monoclonal antibody mixture was added to coated test and control wells. Radioiodinated anti-mouse immunoglobulin was used as the detection antibody.

In the direct monoclonal antibody test, wells were coated with purified 3F7. The use of either gel saline or an irrelevant monoclonal antibody (anti-rat T cell) in the control wells yielded comparable results. Stool specimens were added to test and control wells, and ^{125}I-labeled SR3 globulin served as the detection antibody. None of the stool specimens tested by this assay showed reactivity above background in control wells.

Although both tests served to identify human rotavirus in clinical specimens, the direct monoclonal antibody test was preferable to the multideterminate assay. Six of 87 (7%) positive stool specimens showed an apparent "prozone" effect by the multideterminate assay, i.e., although the initial 2 to 5% stool suspension gave negative or borderline results, further dilutions of the specimen gave positive results. Interference by a prozone effect was not encountered in the direct test. The phenomenon is illustrated in Table 1. When the results of the tests on diluted specimens are considered, both monoclonal antibody tests identified the same 87 specimens as rotavirus positive. However, due to the necessity of titrating test samples, the multideterminate assay was not considered practical and was not studied further.

Comparison of the direct monoclonal assay with Rotazyme. One hundred seventy-seven stool specimens from children with acute diarrheal illness were tested in parallel for the presence of rotavirus by both the direct monoclonal assay and Rotazyme (Fig. 2). Eighty-one specimens were positive and 89 samples were negative by both tests. Of the remaining seven discrepant specimens, six were positive by the monoclonal antibody test and negative by Rotazyme, and one specimen was Rotazyme positive and negative by the monoclonal assay (Table 2). For these seven stool samples, both tests were repeated and the results remained unchanged. The seven discrepant specimens were examined in an independent laboratory under code by direct EM; rotavirus was visualized in four of the specimens, all of which were positive by the monoclonal antibody test but negative by Rotazyme (Table 2, specimens 1 to 4). The remaining three samples were found to be negative by both direct and immune EM. In the specimen that was monoclonal assay negative but Rotazyme positive, no rotavirus was observed by direct and immune EM (Table 2, specimen 7). The two

TABLE 1. Illustration of prozone effect noted with multideterminate assay using monoclonal antibody

Stool specimen	Dilution of stool suspension	P/N ratio	
		Multideterminate assay	Direct monoclonal assay
A	Undiluted	1.3	90.0
	1:4	2.7	39.4
	1:16	4.6	42.6
B	Undiluted	1.8	178.4
	1:4	3.1	66.8
	1:16	6.5	68.4
C	Undiluted	1.6	34.0
	1:4	2.3	33.0
	1:16	5.7	ND[a]

[a] ND, Not determined.

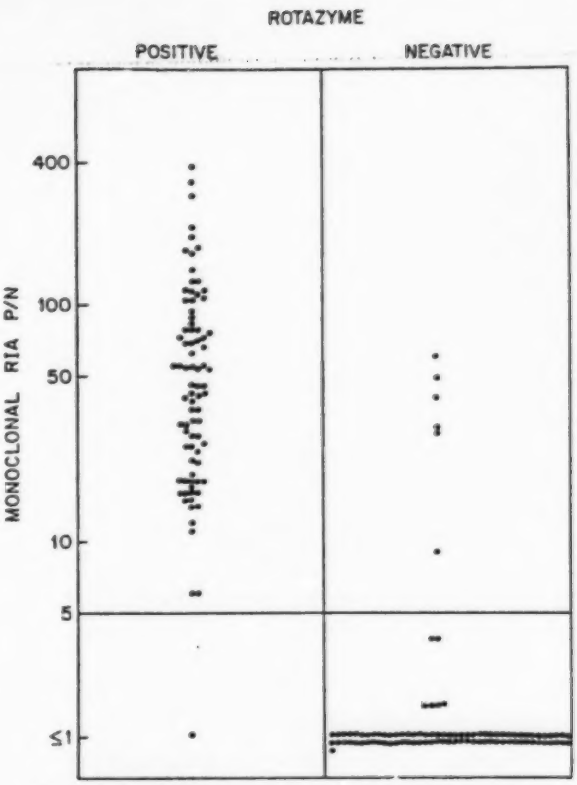

FIG. 2. Distribution of positive-to-negative (P/N) ratio values in direct monoclonal RIA compared with results of Rotazyme for 177 pediatric stool samples. Samples exhibiting P/N values greater than 5 were considered positive by RIA.

EM-negative samples that were monoclonal assay positive and Rotazyme negative were subjected to a confirmatory blocking test with anti-SA11 guinea pig serum and were found to be rotavirus positive (Table 2, specimens 5 and 6).

DISCUSSION

We have presented a new, rapid diagnostic test for rotavirus in human stool specimens that can be readily completed in 3.5 h. The test was designed in our laboratory as a radioimmunoassay but should be readily adaptable to other labeled antibody techniques such as EIA or an avidin-biotin system (32) since only the labeling of conventional rabbit IgG is involved.

The key diagnostic reagent in this test is monoclonal antibody 3F7, which is directed against the common group-specific rotaviral antigen. This monoclonal antibody shows high reactivity with rotavirus of human (Wa), simian (SA11), murine (EDIM), and bovine (NCDV) origin. By immunoprecipitation, 3F7 reacts with VP6, the inner capsid 42,000-molecular-weight structural protein product of the sixth rotaviral gene (1, 21, 26). Subgroup specificity and the common rotaviral antigen are known to be two different antigenic regions of VP6 (13). To improve our chances of selecting a monoclonal antibody group rather than subgroup specificity, we immunized with EDIM, which cannot be classified as either subgroup 1 or 2 (13). The high reactivity of 3F7 with both subgroup 1 (SA11 and NCDV) and subgroup 2 (Wa) rotavirus is indicative of its group specificity

and apparent lack of subgroup prejudice. Previously studied monoclonal antibodies to VP6 (13, 25) have been selected for their subgroup specificity and were useful in subgrouping of rotavirus isolates. The present study is the first report of the use of a characterized monoclonal antibody as a clinical diagnostic reagent for rotavirus in stools.

We compared our new assay with the well-established and widely used Rotazyme test. There was concordance between the two tests in 170 (96%) of 177 specimens. Four of the seven discrepant specimens (Table 2) were clearly resolved in favor of the monoclonal antibody test by visualization of rotavirus particles upon EM examination of the stool samples under code. Two additional discrepant stool specimens that were positive by the monoclonal antibody assay were found to be negative by direct and immune EM, but both were clearly positive by a confirmatory blocking test. The remaining discrepant specimen was Rotazyme positive and was negative by direct and immune EM.

These findings are highly promising in that they indicate the high sensitivity and specificity of the monoclonal antibody test. It is noteworthy that the 3F7 monoclonal antibody is highly reactive as a diagnostic reagent and it more clearly distinguished between positive and negative stool specimens than did our previously described RIA procedure for detection of rotavirus (8). However, based on previously described problems with rotavirus diagnosis (6, 33), it is likely that more specimens, over a period of time and from additional geographical locations, will have to be examined before the monoclonal assay can be evaluated conclusively. Since our study population did not include neonates, it will be of special interest to determine whether the monoclonal antibody test can eliminate the problems of nonspecificity encountered by Rotazyme in diagnosing specimens from this group of patients (18).

TABLE 2. Analysis of seven stool specimens yielding discrepant results with Rotazyme and direct monoclonal antibody assay

Stool specimen	Rotazyme			Monoclonal antibody test				Result of direct EM	Result of immune EM	Blocking test			
	Optical density	Cutoff value[a]	Result	No. of test cpm	No. of control cpm	P/N ratio	Result			With pre-immunization guinea pig serum		With post-immunization guinea pig serum		Result of blocking
										Cpm	P/N ratio	Cpm	P/N ratio	
1	0.031	0.118	−	4,651	115	40.4	+	+						
2	0.029	0.118	−	992	36	27.6	+	+						
3	0.092	0.126	−	2,167	36	60.2	+	+						
4	0.094	0.118	−	344	37	9.3	+	+						
5	0.044	0.105	−	2,004	66	30.4	+	−	−	3,920	45.6	98	1.1	+
6	0.050	0.105	−	1,772	36	49.2	+	−	−	2,482	29.2	77	0.9	+
7	0.172	0.121	+	38	159	0.2	−	−	−					

[a] Lower limit of positivity.

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